

Combination Effect of *N*-Hexane Extract of *Plectranthus amboinicus* (Lour.) Spreng. with Doxorubicin Againsts HeLa Cell Lines

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Abstract

The study was aimed to investigate the growth inhibiting effect of *Plectranthus amboinicus*, (Lour.) Spreng *n*-hexane extract (PAN) in combination with doxorubicin againsts HeLa cell Lines, to observe the apoptotic induction and immunocytochemistry of HeLa cell Lines on cyclin D1, Bcl-2, and Cox-2 after treatment of PAN. The percentage viability of the cell were carried out by using MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide] assay. The effect of apoptosis was observed by flowcytometry assay in single dose of PAN. The expression of cyclin D1, Bcl2 and COX-2 proteins were investigated on HeLa cell lines by using immunocytochemistry. The result showed that PAN showed strong synergistic effect with doxorubicin againsts HeLa cells based on Combination Index analysis. No significant apoptotic induction of PAN in HeLa cell lines, but the extract caused necrosis. The immunocytochemical study showed suppression of cyclin D1, Bcl₂ and COX-2 expression on HeLa cell lines. The results concluded that PAN could be a potential co-chemotherapeutic agent with doxorubicin on cervix cancer cells but need to be explored further by its combination on specific molecular target.

Keywords : *Plectranthus amboinicus*, (Lour.) Spreng., combination, HeLa cell lines

INTRODUCTION

The latest world report provides clear evidence that global cancer rates could increase to 15 million by 2020 and is expected to grow to 21.4 million new cancer cases and 13.2 million cancer deaths by 2030. Cancer is one of the most life-threatening diseases with more than 100 different types. Due to lack of effective drugs, expensive cost of chemotherapeutic agents and their side effects, cancer can be a cause of death. Conventional cancer therapies, including surgery and chemotherapy have a limited but important role in the overall treatment of most solid tumors. Chemotherapy drugs are not selective in killing cells because they affect the synthesis of nucleic acids and protein, so that normal body cells also die (MacDonald, *et al.*, 2004).

Doxorubicin as chemotherapeutic agent causes serious problems such as drug resistance and toxic effect on normal tissue especially on heart. The manifestations are heart failure,

hypertension, oedema, bradychardi, thromboembolism. Thus the strategies of cancer treatment using combined therapies or combined agents are considered more promising for higher efficacy, and become alternative, resulting in a better survival.

Plant have been source of medicine for thousands of years and phytochemicals continue to play an essential role in medicine. One of the medicinal plant is *Plectranthus amboinicus* (Lour.) Spreng. The previous studies had showed that the *n*-hexane, ethylacetate and ethanol extracts of *Plectranthus amboinicus*, (Lour.) Spreng. had antioxidant activities. The *n*-hexane and ethylacetate extracts exhibited strong cytotoxic effect on T47D breast cancer cells with IC₅₀ value of 44.716 µg/mL and 37.61 µg/mL, respectively. Antioxidant activity is usually correlated with cancer prevention.

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Thus, the extract has potential effect as a chemoprevention. The cytotoxic effect of *n*-hexane, ethylacetate and ethanol extracts were also examined on the HeLa cell lines. The study showed that the three extracts had cytotoxic effect on HeLa cell with IC₅₀ values 76.322 µg/mL, 143.291 µg/mL, and 88.997 µg/mL, respectively. The aims of this research are to investigate cytotoxic activity of PAN-doxorubicin combination, to analyze apoptotic induction and the proteins expression of HeLa cell lines after single treatment of PAN.

MATERIALS AND METHODS

Plant Material

Fresh leaves of *Plectranthus amboinicus*, (Lour.) Spreng. was collected from Pematang Siantar, Simalungun regency, Sumatera Utara province, Indonesia. *Plectranthus amboinicus*, (Lour.) Spreng. was identified in Research Centre for Biology, Indonesian Institute of Science, Bogor, and the voucher specimen was deposited in herbarium.

Preparation of *n*-Hexane Extract (PAN)

The air-dried and powdered leaves of *Plectranthus amboinicus*, (Lour.) Spreng. (1 kg) were repeatedly extracted by cold maceration with *n*-hexane (3x3 d, 7.5 L). The powder were dried in the air and extracted with ethylacetate (3x3 d, 7.5 L) at room temperature on a shake. The filtrate was collected, and then evaporated under reduced pressure by rotary evaporator (Heidolph VV-200) to obtain a viscous extract and the concentrated extract was dried by freeze-dryer (Edwards).

Chemicals: *n*-hexane and ethylacetate were purchased from Merck (Darmstadt, Germany), DMSO (Sigma Aldrich Chemie GmbH Germany), [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) (Sigma Chemical, St. Louis, MO), RPMI media and Phosphate Buffer Saline (FBS) 10% v/v (Gibco, Grand Island, NY, USA), Doxorubicin (Ebewe).

Cytotoxicity Assay

Cytotoxicity was determined by the MTT assay. Briefly, HeLa cells were plated at 10⁴ cells/well in a 96-well plate. After incubation for 24 h at 37°C, cells were treated by *Plectranthus amboinicus n*-hexane extract (PAN) with different concentration and incubated for 24 h. MTT solution was added to each well and further incubated for 4h at 37°C, optical density was read with an ELISA reader at 595 nm.

Flowcytometry Assay Apoptosis Assay

HeLa cells (5x10⁵ cells/well) were seeded into 6-well plate and incubated for 24 h. After that, the cells were treated with PAN, and then incubated for 24 h. Both floating and adherent cells were collected in conical tube using trypsin 0.025%. The cells were washed thrice with cold PBS and centrifuged 2500 rpm for 5 min. The supernatant was separated, while the sediment was collected and fixed in cold 70% ethanol in PBS at -20°C for 2 h. The cells were washed thrice with cold PBS and centrifuged in 2500 rpm for 5 min. The supernatant was separated, while the sediment was collected and fixed in cold 70% ethanol in PBS at -20°C for 2 h. The cells were washed thrice with cold PBS then suspended then centrifuged 3000 rpm for 3 min and Annexin V kit added to sediment and suspended and incubated at 37°C for 30 min. The samples were analyzed using FACScan flowcytometer.

Immunocytochemistry

HeLa cells (5x10⁴ cells/well) were seeded on coverslips in 24-well plate and incubated for 24 h. After that, the cells were treated with PAN, and then incubated for 24 h. After incubation, the cells were washed with PBS and then fixed with cold methanol at 4°C for 10 min. After that, the cells were washed with PBS and blocked in hydrogen peroxide blocking solution for 10 min at room temperature, incubated using primary antibody Bcl-2, cyclin D1, and COX-2 for 1 h, then washed thrice with PBS, then incubated with secondary antibody for 10 min. The cells were

washed with PBS, then incubated in 3,3-diaminobenzidine (DAB) solution for 10 min, and washed with aquadest. Afterward, the cells were counterstained with Mayer-Haematoxylin for 5 min, and the coverslips were taken and washed with aquadest, and then immersed with xylol and ethanol 70%. Protein expression observed by light microscope (Nikon YS100). Cells that express a particular protein will provide the brown colour, while the cells that does not give a specific protein will provide blue colour.

RESULTS AND DISCUSSIONS

The aims of the study were to investigate the efficacy of PAN as a co-chemotherapy on doxorubicin treatment, to analyze apoptotic induction and proteins expression of HeLa cell lines after treatment of the extract. PAN, doxorubicin and their combination were investigated for their cytotoxicity effect on HeLa cell lines. Cell viability was determined by MTT method after incubation for 24 h, and the effect of combination was analyzed by Combination Index analysis. The *Combination Index* (CI) analysis is one of the most popular method for evaluating drug interactions in combination cancer chemotherapy. The method is used to categorize the effect of the combination which is synergistic, additive, or antagonis. In every treatment (PAN and their combination) was showed the inhibition of cells growth. The IC_{50} value of PAN 76.322 $\mu\text{g/mL}$ and doxorubicin 1.8 $\mu\text{g/mL}$, and the combination was showed higher inhibitory effect if compare with single treatment. The

optimum combination index (synergistic effect) was showed in $1/4$, $3/8$, and $1/2$ IC_{50} value of PAN and $1/2$ IC_{50} value of doxorubicin (0.6 $\mu\text{g/mL}$) categorized with strong synergistic effect ($CI < 0.1$). These effects supposed to be related to apoptotic induction and expression of some proteins.

In this study, the apoptotic induction and expression of proteins were done at PAN single treatment, because we wanted to know its effect alone. The effect of its combination with doxorubicin need further study. Evaluation of apoptotic induction was performed by using flowcytometry assay with Annexin V. As shown in Fig. 1, the cells in the upper and lower right quadrants represent late apoptotic/necrotic and early apoptotic cells, respectively. The percentage of PAN, in early apoptotic was 0.20%, in late apoptotic/ early necrotic 11.04% and in late necrotic 79.03%.

The expression of apoptosis regulator protein Bcl₂, the protein that play important role in cell cycle, Cyclin D1, and expression of COX-2 which contribute to the development of human cancer, were observed in HeLa cells by using PAN treatment. COX-2 derived prostaglandin E2 induces angiogenesis of tumor development by increasing of angiogenic factors, or decreased expression of anti-angiogenic factors, or a combination of both event. The effect of PAN on cyclin D1, Bcl2 and COX-2 expressions were observed by using immunocytochemistry. Expression of cyclin D1, Bcl2 and COX-2 proteins are positive characterized by brown stained nuclei in the cells (Fig. 2).

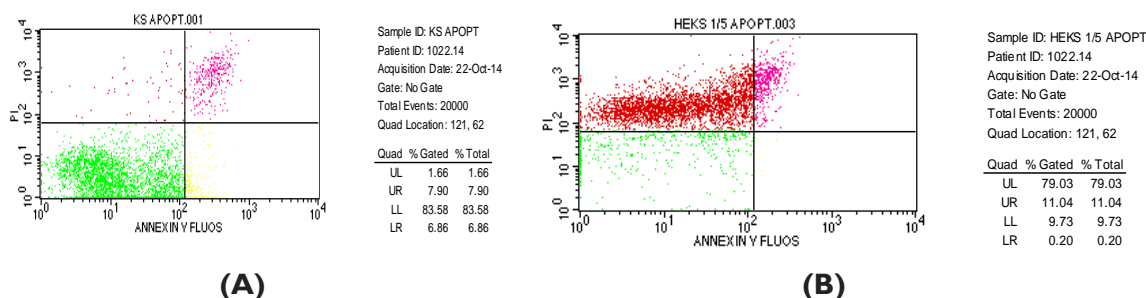


Figure 1. Apoptotic analysis of PAN on HeLa cell lines. (A) control cells; (B) PAN 1/5 IC_{50} (15.26 $\mu\text{g/mL}$)

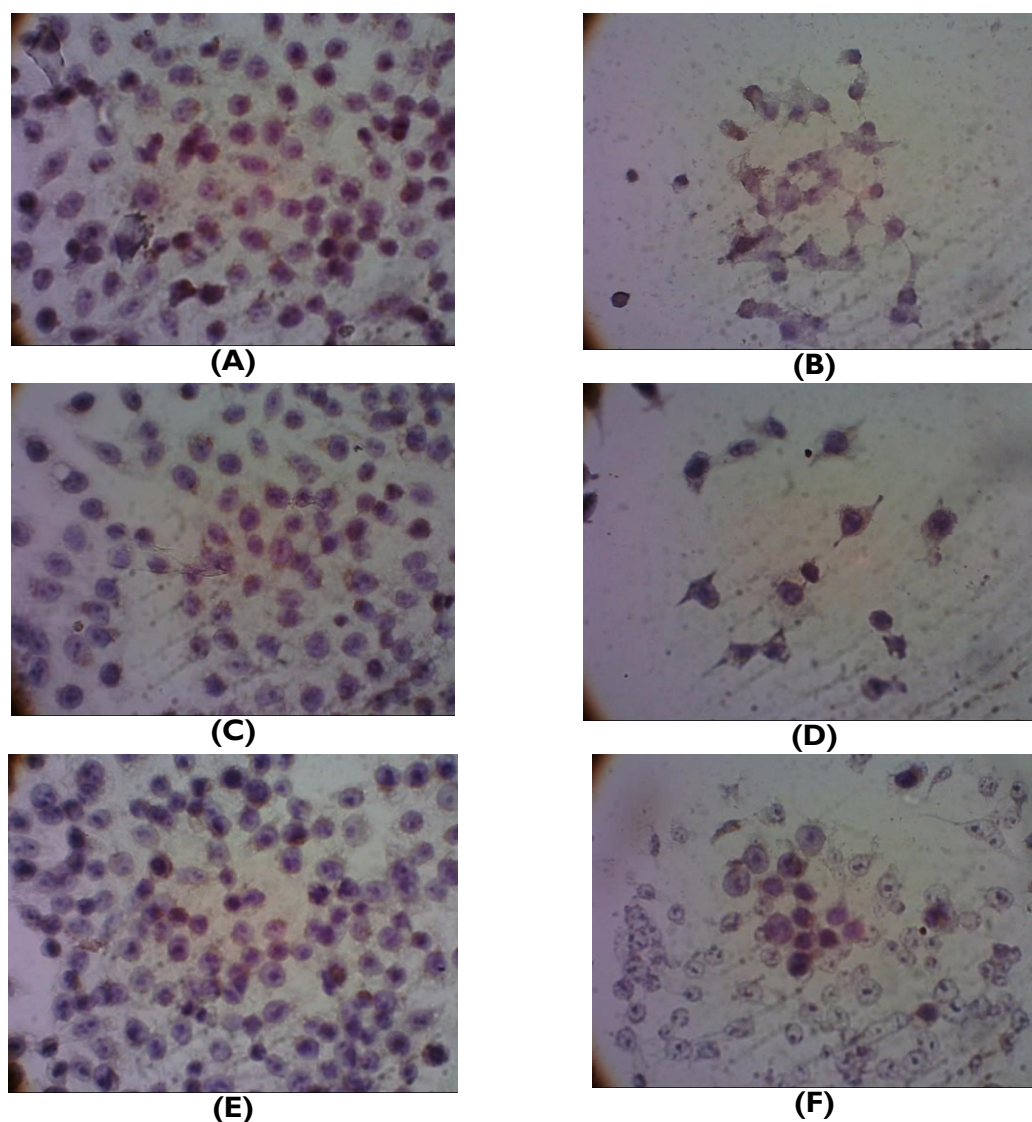


Figure 2. Expression of Bcl2, cyclin D1, and COX-2 after treatment of PAN 1/5 IC_{50} (15.26 μ g/mL) observed by immunocytochemistry assay. (A) HeLa control cell; (B) Bcl2 expression; (C) HeLa control cell; (D) Cyclin D1 expression; (E) HeLa control cell; (F) COX-2 expression.

The observation of apoptosis regulator protein Bcl2 was conducted in HeLa cells after treatment of PAN. Immunocytochemistry assay with Bcl2 antibody showed the expression of Bcl2 was decreased by PAN, therefore it is strengthen the apoptosis mechanism of PAN. One of secondary metabolite in *Plectranthus amboinicus* is ursolic acid. Ursolic acid inhibit EGFR/MAPK and suppress Bcl2 expression on colon cancer cell by activation caspase 3 and 9. Thus, it is possibly that PAN could induce

apoptosis through the same pathway, but we need further study. As seen in Fig. 2, the untreated cells (control) showed high intensity for Bcl2, cyclin D1 and COX-2. A single treatment of PAN was decreased on Bcl2, cyclin D1 and COX-2 expression. Inhibition of cyclin D1 protein expression strengthen the mechanism of modulating cell cycle especially in inhibition of cell cycle on G_0 - G_1 phase. Cyclin D1 play important role in G_0 - G_1 phase with established complex with CDK-4 or CDK-

6 to controlled G₁ to S phase transition. However, we need to be explored more detail about the molecular mechanism of apoptosis induction, cell cycle modulation, and antiangiogenic regulation of PAN. Based on the results, it can be concluded that combination of *n*-hexane extract of *Plectranthus amboinicus* (Lour.) Spreng. leaves and doxorubicin synergistically inhibit the HeLa cell lines.

CONCLUSION

Based on the immunocytochemistry assays, the *n*-hexane extract of *Plectranthus amboinicus* can be used in development of chemopreventive agent on cervical cancer.

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